



Microbial community dynamics of a blue-veined raw milk cheese from the UK

Journal:	<i>Journal of Dairy Science</i>
Manuscript ID	JDS-17-14104.R1
Article Type:	Research
Date Submitted by the Author:	n/a
Complete List of Authors:	Yunita, Dewi; Universitas Syiah Kuala Fakultas Pertanian, Teknologi Hasil Pertanian Dodd, Christine; University of Nottingham, Food Sciences;
Key Words:	raw milk, blue-veined cheese, PCR DGGE, microbial diversity

SCHOLARONE™
Manuscripts

BLUE-VEINED RAW MILK CHEESE MICROBIAL COMMUNITY

1 **Interpretive Summary**

2 Microbial community dynamics of a blue-veined raw milk cheese from the UK. By
3 Yunita and Dodd. Raw milk may introduce many microbial species into a cheese which
4 could be important for product characteristics. We show that whilst some raw milk
5 species are lost during cheese fermentation and ripening, some survive. Salt addition and
6 handling introduce new species contributing mainly to rind microbiota. In blue-veined
7 cheeses, piercing introduces these bacteria from the surface to the core but these often
8 fail to establish.

9
10 **Title**

11 Microbial community dynamics of a blue-veined raw milk cheese from the UK

12
13 Dewi Yunita,^{*†1} and Christine E. R. Dodd^{*}

14
15 ^{*}Division of Food Sciences, School of Biosciences, University of Nottingham, Sutton
16 Bonington Campus, Loughborough, Leicestershire LE12 5RD, United Kingdom.

17 [†]Department of Agricultural Product Technology, Faculty of Agriculture, Syiah Kuala
18 University, Darussalam, Banda Aceh 23111, Indonesia.

19
20 ¹Corresponding author: dewi_yunita@unsyiah.ac.id

21
22 Running title: Blue-veined raw milk cheese microbial community

23

ABSTRACT

A commercial blue-veined cheese made from unpasteurized milk was examined by conventional culturing and PCR Density Gradient Gel Electrophoresis analysis of the bacterial community 16S rRNA genes using three primer sets V3, V4V5, V6V8. Genomic DNA for amplification was extracted directly from raw milk, starter culture, cheese at different stages of production, fully ripened cheese and from the cultured cells grown on various media. The outer **rind** was sampled separately from the inner white core and blue veins. A diverse microbiota containing *Lactococcus lactis* subsp. *lactis*, *Lactobacillus plantarum*, *Lactobacillus curvatus*, *Staphylococcus gallinarum*, *Staphylococcus devriesei*, *Microbacterium* sp., *Sphingobacterium* sp., *Mycetocola* sp., *Brevundimonas* sp., *Enterococcus faecalis*, *Proteus* sp. and *Kocuria* sp. was detected in the raw milk using culturing methods, but only *Lactococcus lactis* subsp. *lactis*, *Lactobacillus plantarum* and *Enterococcus faecalis* survived into the final cheese and were detected both in the core and the **rind**. Using PCR Density Gradient Gel Electrophoresis analysis of the cheese process samples, *Staphylococcus equorum* and *Enterococcus durans* were found in the **rind** of pre-piercing samples but not in the core and veins; after piercing, these species were found in all parts of the cheese but survived only in the **rind** when the cheese was fully ripened. *Brevibacterium* sp., *Halomonas* sp., *Acinetobacter* sp., *Alkalibacterium* sp. and *Corynebacterium casei* were identified only by PCR Density Gradient Gel Electrophoresis and not cultured from the samples. *Brevibacterium* sp. was initially identified in the cheese post piercing (core and veins), *Halomonas* sp. was found in the matured cheese (**rind**), *Acinetobacter* sp., *Alkalibacterium* sp. and *Corynebacterium casei* were also in the pre-piercing samples (**rind**) and then found through the subsequent process stages. The work suggests that in

48 this raw milk cheese, there is a limited community from the milk surviving into the final
49 cheese, with salt addition and handling contributing to the final cheese consortium.

50

51 **Key words:** raw milk, blue-veined cheese, PCR DGGE, microbial diversity

For Peer Review

INTRODUCTION

The most strictly prescribed unpressed blue-veined cheese in the United Kingdom (UK) is Stilton. It is a protected designation of origin (PDO) product of the counties of Derbyshire, Leicestershire and Nottinghamshire, with the requirement that milk is pasteurized at the first stage. Since March 1990, a blue-veined raw milk cheese made by the same process as Stilton has been produced commercially in Nottinghamshire. This cheese has the same texture and appearance as Blue Stilton which has a creamy white curd, open texture with blue-green well-distributed veins (created by piercing the cheese during ripening) and rough brown rind (Scott et al., 1998). Both cheeses use similar lactic cheese starter cultures which consist of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*. Both cheeses also contain a starter *Penicillium roqueforti* mold which starts to grow in the core of the cheese after the piercing process, due to the aerobic conditions this creates, giving the blue-vein appearance of the cheese which develops during the ripening period (Bockelmann, 2010).

The microbial community of Stilton has been previously studied and shown to present a complex consortium of bacteria (Ercolini et al., 2003) and yeasts (Gkatzionis et al., 2014). This non-starter microbiota varies in the different parts of the cheese (white core, blue veins and outer rind) and has been shown to contribute to the flavor volatile production, which also shows variation in the different regions of the cheese (Gkatzionis et al., 2013, 2014; Price et al., 2014). However, there is no published work on raw milk blue-veined cheese originally from the UK. This may differ as the microorganisms in the raw milk will be different, coming from the cow and milking

75 environment, and these may contribute to the final cheese community and determine
76 sensory properties.

77 There is much debate amongst cheese makers on the use of raw or pasteurized
78 milks for cheese making. The risks and benefits for traditional cheeses, mainly raw milk
79 cheeses, has been discussed by Montel *et al.* (2014). The concern over use of
80 unpasteurized milk in cheese production is related to health concerns caused by
81 pathogenic bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella*
82 and *E. coli* found in cheeses (Lafarge and Lahellec, 2000). Pasteurization is usually used
83 to assure the safety of milk and is the reason why Stilton cheese makers use pasteurized
84 milk. However, for raw milk cheese makers, pasteurization kills desirable bacteria and
85 destroys enzymes and proteins resulting in a less tasty cheese. The problem of raw milk
86 safety risks can be addressed, as the fermentation should kill pathogenic bacteria if it is
87 done properly. So, the main concern of traditional cheese producers is to preserve
88 microbial diversity and exploit its benefits.

89 Therefore, in the current study, we have analyzed the bacterial diversity during
90 raw milk blue-veined cheese production using a traditional culture approach and 16S
91 rRNA gene polymerase chain reaction denaturing gradient gel electrophoresis (PCR
92 DGGE) techniques to evaluate the bacteria which contribute to production of the
93 characteristics of the product. The presence of *Lactococcus lactis* at the end of
94 production has also been examined by pulse-field gel electrophoresis (PFGE) to
95 determine whether its origin was from the raw milk or the added starter cultures. This
96 should create an understanding of raw milk cheese production and the microbiota
97 components which are needed to allow cheese makers to make a consistent product.

98

MATERIALS AND METHODS

Sampling

The UK blue-veined raw milk cheese is made from unpasteurized milk. The process is started by pouring the milk into a stainless steel tank, heating up to 30°C and adding starter cultures (*Lactococcus lactis* and *Penicillium roqueforti*) and 90 minutes later adding rennet. The mixture is fermented at around 30-40°C. After 6 hours, the curds form and are cut by hand into small cubes to release the whey. The soft curds then are transferred manually by ladles into a second tank and left overnight. The curds are then milled, salted and poured into plastic cylindrical hoops without pressing. Then, the cheeses are left in the hastener (21°C) and turned daily for 5 days to allow the whey to drain. On the fifth day, the cheeses are removed from the hoops and the outside smoothed by knives in a process termed 'rubbing up'. The cheeses are then moved to the ripening rooms (T = 13°C; RH = 85%) and six weeks later, fresh cheese is pierced by stainless steel needles to allow development of the blue veins. Finally, after around another 6 weeks (T = 13°C; RH = 90%) the cheeses are fully matured.

Samples from bulk raw milk, frozen starter culture and a raw milk blue-veined cheese during production were taken aseptically in the spring season in Nottinghamshire. The production sites sampled were: pre-milling, post-milling and salting, pre-piercing (6 weeks), post-piercing (9 weeks) and fully ripened cheese (12 weeks). The raw milk sample was processed immediately after collecting, but the frozen starter culture was left overnight at room temperature (at around 20°C) to thaw following the manufacturer's procedure for use in cheese production. The rest of the samples were analyzed within 6 h or kept cool at 4°C for no longer than 24 h. For all cheese samples, the outer rind was separated from the inner core of the cheese and the two regions were

123 tested separately. The inner core was collected using a sterilized cheese corer. All
124 samples were obtained in triplicate.

125

126 *Microbiological Analysis*

127 The cheese samples (25 g) were weighed into a stomacher bag and diluted in
128 quarter-strength Ringer's (225 ml; Oxoid, Hampshire, UK) and homogenized in a
129 stomacher at 230 RPM for 2 minutes (Stomacher 400 Circulator, Seward, West Sussex,
130 UK). These samples were considered as the 10^{-1} dilution and were further diluted to 10^{-8}
131 by tenfold serial dilutions in the same diluent. Milk and starter culture samples were
132 directly diluted in quarter-strength Ringer's. Samples (0.1 ml) of each dilution were
133 spread-plated in triplicate on non-selective and selective media. Mesophilic aerobic
134 bacteria were counted on brain heart infusion (**BHI**) agar, yeasts and molds on rose
135 bengal chloramphenicol agar (**RBCA**), lactococci on M17 agar, lactobacilli on Rogosa
136 agar, lactic acid bacteria (**LAB**) on MRS agar, enterococci on KF Streptococcal agar
137 (**KFSA**) and staphylococci on Baird Parker (**BP**) agar. All agars were from Oxoid. All
138 bacterial plates were incubated at 30°C for 2 days while yeasts and molds were grown at
139 25°C for 5 days. LAB and lactobacilli were incubated under anaerobic conditions which
140 were obtained by using AnaeroGen Gas Pack (Oxoid) (Conte et al., 2011). Presumptive
141 *Staphylococcus aureus* from BP agar were confirmed by catalase (using 40% H₂O₂) and
142 coagulase tests (Staphytect Plus Test, Oxoid) (Collins et al., 1995).

143

144 *Selection and Characterization of Isolates*

145 To analyze the cultured populations, a total of 80 isolates (41 isolates from the
146 mature cheese and 39 isolates from raw milk) with different colony types were randomly

147 picked from the seven media used. Each isolate was purified by streak-plating three
148 times and stored in Microbank™ Bacterial and Fungal Preservation System (Pro-Lab
149 Diagnostic, Wirral, UK) at -80°C. Isolates were examined for cell morphology by Gram
150 staining and for their ability to grow at 10°C for 4 days and 40°C for 2 days.

151

152 ***DNA Extraction***

153 Bulk cell suspensions were collected from the culture plates by adding 1 ml of
154 sterile phosphate buffered saline (Oxoid) onto the confluent growth plates (10^{-1}
155 dilution). A DNeasy® Mericon Food Kit (Qiagen) was used to extract DNA from cheese,
156 milk, and bulk cell suspensions. The following procedure was used with some
157 modification of the manufacturer's instructions. Briefly, the cheese sample (200 mg),
158 milk sample (0.2 ml) or bulk cell suspension (0.2 ml) was placed into a sterile 1.5 ml
159 microcentrifuge tube. Then, 1 ml of Food Lysis Buffer (cetyltrimethylammonium
160 bromide, CTAB) and 2.5 µl of proteinase K solution were added. The mix was
161 incubated for 30 min at 60°C in a thermomixer (Eppendorf, Germany) at 1,000 RPM
162 followed by cooling for 1 min and centrifuging at 10,000 g for 5 min. The clear
163 supernatant (700 µl) was placed into a new tube containing 500 µl of chloroform (Fisher
164 Scientific, UK). Then, the mix was vortexed for 15 s and centrifuged at 14,000 g for 15
165 min. The upper solution (250 µl) and 1 ml of Buffer PB (5M guanidine hydrochloride
166 and 30% isopropanol) were mixed thoroughly in a new tube by vortexing then
167 transferred into the QIAquick spin column prior to centrifuging at 17,000 g for 1 min.
168 Buffer AW2 (a low salt buffer, 500 µl) was added onto the column and this was
169 centrifuged at 17,000 g for 1 min. The flow-through was discarded and the column was
170 centrifuged again at 17,000 g for 1 min. Finally, the column was transferred into a new

171 tube and 100 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) was added. The mix was
172 incubated at room temperature for 1 min before it was centrifuged at 17,000 g for 1 min
173 to elute the DNA.

174 DNA was extracted from reference strains (*Lactococcus lactis* NCIMB 9918,
175 *Lactococcus lactis* NCIMB 8763, *Lactobacillus plantarum* NCIMB 138914,
176 *Enterococcus faecalis* NCTC 775 and *Leuconostoc mesenteroides* (University of
177 Nottingham, Division of Food Sciences strain collection) and selected pure isolates from
178 cheese by a boiling procedure used to lyse the cells in a heating thermocycler. The
179 procedure followed Cocolin et al. (2001).

180 The extracted DNA (2 µl) concentration and purity were measured at a
181 wavelength of 260 nm by Nanodrop (ND-1000 spectrophotometer, Thermo Scientific,
182 Wilmington, USA). A ratio of absorbance at 260 nm and 280 nm (260/280) of 1.8 is
183 generally accepted as pure for DNA as lower ratios indicate the presence of protein,
184 phenol or other contaminants (Nanodrop Technologies, Inc., 2007). The extracted DNA
185 (1.5 µl) was then used as template in PCR reactions.

187 **PCR Amplification**

188 The V3, V4V5, V6V8 regions of 16S rRNA genes were amplified by PCR as
189 previously reported (Muyzer et al., 1993; Nübel et al., 1996; Schwieger and Tebbe,
190 1998). For DGGE analysis, a GC-clamp was added to each forward primer (Muyzer et
191 al., 1993). PCR amplification was performed with DreamTaq™ Green PCR Master Mix
192 (2x) (Fermentas, Yorkshire, UK) in a programmable heating thermocycler (Biorad
193 C1000™ Thermal Cycler, Hertfordshire, UK). Nuclease-free water (23.3 µl;
194 Fermentas), 0.1 µl of each primer (MWG-Biotech, Ebersberg, Germany) and 1.5 µl of

sample DNA were added into the master mix (25 μ l) to give a final volume of 50 μ l. The PCR product (5 μ l) was checked by electrophoresis on a 1% TAE agarose gel (Agarose 3:1, Melford Laboratories Ltd., Suffolk, UK) containing ethidium bromide (0.2 μ g/ml) in 1X TAE running buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) at 75 V for about 45 min. The size of the amplified DNA was determined against a 100 bp molecular weight marker (Promega, Southampton, UK). The gel was imaged under a UV transilluminator (GelDoc XR, BioRad) and gel pictures were recorded using the GelDoc system (The Quantity One 4.6.5 Basic software, USA).

203

204 ***DGGE Analysis***

Samples of 200 bp size PCR products (using V3 primer sets) were analyzed in 8% (w/v) polyacrylamide gels in 1x TAE buffer while those of 400 bp sizes (using V4V5 and V6V8 primer sets) were run in 6.5% (w/v) polyacrylamide gels (Ercolini et al., 2003). Parallel electrophoresis was performed at 20 V for 10 min followed by 16 h at 50 V by using a Bio-Rad Dcode apparatus (Universal Mutation Detection System, USA). The gel contained 20-80% urea formamide for which 100% denaturant solutions consisted of 40% (v/v) formamide and 7 M urea. All DGGE reagents were from Severn Biotech Ltd., Worcestershire, UK. The gel was stained with GelStar (Lonza Rockland, ME USA) and the images recorded.

214

215 ***Sequencing of DGGE Bands***

Selected bands were excised from the gel using a sterile scalpel and kept at 4°C for 24 h in 20 μ l of TE buffer (10 mM Tris Base, 1 mM EDTA; pH 7) to elute DNA into the buffer. The eluted DNA (1.5 μ l) was reamplified with the same PCR program but

219 without the GC-clamp in the respective forward primer. The PCR products (15 µl) were
220 purified with Zymoclean Gel DNA Recovery Kit (The Epigenetics Company;
221 Cambridge, UK) according to the manufacturer's procedures. Following purification, 15
222 µl of purified DNA was sequenced (MWG Laboratory, Germany). The DNA sequences
223 were compared with those in public data libraries by using BLAST (Basic Local
224 Alignment Search Tool) search program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

225

226 ***Preparation of Lactococcus lactis Isolates for PFGE Analysis***

227 *Lactococcus lactis* was isolated from two commercial starter cultures used
228 alternately by the cheese producers (liquid and powdered) and fully ripened blue-veined
229 raw milk cheese (12 weeks). The added starter cultures contained *Lactococcus lactis*
230 subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*
231 biovar. *diacetylactis*. The liquid starter culture was used directly for isolation, while
232 powdered starter culture was prepared in UHT milk which had been heated at 32°C for
233 24 h before use following the manufacturer's procedure; 0.1 g starter culture (OV100,
234 Orchard Valley Dairy Supplies, Worchestershire, UK) in 10 ml heated milk. For the
235 cheese, the rind was also tested separately from the core and blue veins. All samples of
236 each dilution were spread plated (0.1 ml in duplicate) and grown aerobically on M17
237 agar (Oxoid CM 0785) and anaerobically (AnaeroGen kit; Oxoid AN0035A) on MRS
238 agar (Oxoid CM 0361) at 30°C for 2 days. Twenty five isolates were picked from each
239 sample on each media agar and were restreaked twice to get pure isolates for further
240 characterization. Isolates with the following characteristics: Gram positive cocci,
241 catalase negative, oxidase negative and non-group D were presumptively identified as
242 *Lactococcus lactis* isolates and were confirmed by sequencing of V3 region.

243 *Lactococcus lactis* subsp. *lactis* NCIMB 6681, *Lactococcus lactis* subsp. *cremoris*
244 NCIMB 8662 and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* NCIMB 10484
245 were used as controls. These strains were grown in MRS broth (Oxoid CM0359) at 30°C
246 and 100 RPM (Gallenkamp) for 24 h.

247

248 ***Preparation of Digested Genomic DNA for PFGE Analysis***

249 Cells were harvested from 3 ml of an overnight culture grown in BHI broth at
250 30°C and 100 RPM agitation (Gallenkamp), by centrifugation (Eppendorf) at 13,000 g
251 for 60 s. In a microcentrifuge tube, the cell pellet was re-suspended and washed twice
252 with 1 ml of TN buffer [1M NaCl (Fisher Scientific S/316/60) and 10 mM Tris-HCl
253 (Sigma T6066)] at pH 6.7 by centrifugation (Hettich Zentrifugen Mikro200) at 13,000 g
254 for 60 s. Re-suspended cells (300 µl) in TN buffer were warmed to 55°C (Thermomixer
255 Eppendorf) and mixed with 300 µl of 1% (w/v) pulse-field certified agarose (Bio-Rad
256 161-3109) made up in TN buffer. The suspension mixture was poured into a CHEF plug
257 mould (Bio-Rad) and allowed to solidify for 15 min at room temperature (20°C). The
258 embedded cells were lysed with 4 ml of lysis buffer [6 mM Tris-HCl, 0.1 M EDTA
259 (Fisher Scientific D/0700/53) and 1% (w/v) N-lauryl-sarcosine sodium salt (Sigma
260 L9150), pH 7.6] containing 10 mg/ml lysozyme (Sigma L6876) overnight at 37°C. The
261 plugs were washed three times with 3 ml of TE buffer [10 mM Tris-HCl and 1 mM
262 EDTA, pH 7.6] for 30 min at room temperature followed by incubation in 4 ml of
263 proteinase-K buffer [0.5 M EDTA, 1% (w/v) N-lauryl-sarcosine sodium salt, and 1
264 mg/ml proteinase-K (Fermentas EO 0491), pH 8.5] for 24 h at 55°C (100 RPM;
265 Gallenkamp). The plugs were treated twice with 4 ml of TE1 buffer [10 mM Tris-HCl
266 and 1 mM EDTA, pH 8] containing 1 mM phenylmethylsulphonyl fluoride (PMSF;

267 Sigma P7626) shaken at 100 RPM for 1 h at 55°C. The addition of PMSF was to obtain
268 vivid, discrete and clear bands after running the gel (Obszańska *et al.*, 2015). The plugs
269 were finally rinsed three times with TE2 buffer [10 mM Tris-HCl and 50 mM EDTA,
270 pH 8] for 30 min at room temperature. The plugs were stored at 4°C in TE1 buffer until
271 use.

272 Before digestion, the DNA plugs were rinsed in 500 µl sterile distilled water for
273 15 min at room temperature followed by rinsing in 200 µl restriction buffer-D [6 mM
274 Tris-HCl, 6 mM MgCl₂, 150 mM NaCl, 1 mM dithiothreitol, pH 7.9; (Promega
275 R004A)]. The digestion was carried out for 24 h at 37°C in a 100 µl of solution
276 consisting of *Sma*I (0.4 U/µl; Promega R6121), bovine serum albumin (BSA; 0.2 µg/µl;
277 Promega R396D) and buffer J (1X; Promega R009A). The digested plugs were removed
278 from the reaction reagents and stored at 4°C in 0.5X TBE buffer [44.5 mM Tris-HCl,
279 44.5 mM boric acid (Sigma B7901), 1.25 mM EDTA, pH 8] until use.

281 ***PFGE Analysis***

282 The electrophoresis conditions were followed from Obodai (2006) with minor
283 modifications. Electrophoresis was run in 2 l of 0.5X TBE buffer [44.5 mM Tris-HCl,
284 44.5 mM boric acid (Sigma B7901), 1.25 mM EDTA, pH 8] containing 100 µM
285 thiourea, at 14°C for 16 h at 6 V/cm, using a counter-clamped homogenous
286 electrophoresis cell (Bio-Rad). The pulsed time used was 4-45 s and pump pressure was
287 80 RPM. A 50-1000 kb DNA ladder (Sigma D-2416) was used as a molecular size
288 marker. DNA was visualized by staining in 100 ml of sterile distilled water containing
289 10 µl of ethidium bromide (10 mg/ml; Fisher Scientific) for 1 h at room temperature
290 followed by de-staining in 100 ml of sterile distilled water for 30 min at room

291 temperature. The gels were imaged under a UV transilluminator (GelDoc XR, Bio-Rad)
292 and recorded with Quantity One 4.6.5 Basic Gel Doc software (Bio-Rad).

293

294 ***PFGE Cluster and Statistical Analysis***

295 PFGE profiles were saved as 150 dpi tiff format and were analyzed using
296 FPquest version 4.5 software (Bio-Rad). Calculation of the similarity of the band
297 profiles and grouping of the PFGE patterns was based on the Dice correlation coefficient
298 and the unweighted pair group method with arithmetic averages (UPGMA) cluster
299 analysis. Calculation of the significance of the PFGE profiles was done by AMOVA
300 GenAlEx 6.5: Genetic Analysis in Excel.

301

302 **RESULTS**

303 ***Enumeration of Microbial Population by Plate Counts***

304 The bacterial populations of the raw-milk cheese were enumerated through the
305 different stages of production (Table 1); pH values were also recorded at each stage.
306 The pH of the raw milk decreased after starter addition and then changes developed
307 through production. Interestingly, unlike with other mold-ripened cheeses where the pH
308 of the rind is higher than that of the core because of ammonia produced by a high yeast
309 count (Gori et al., 2007; Mounier et al., 2006) and a high mold count (Fox et al., 2004),
310 the higher pH of the rind relative to the core of the mature cheese was not associated
311 with a higher total yeast and mold count (RBCA) and in fact was 2 log₁₀ cfu/g lower.
312 Presumptive *Lactococcus* counts (M17 agar) increased in the early stages of cheese
313 fermentation with higher counts developing in the rind than in the core in later
314 production stages. The mature cheese showed similar results. Yeasts and molds were not

isolated from the raw milk but became evident after milling and salting; these counts increased through the production stages with counts highest following piercing at 9 weeks. Counts in the **rind** were always higher than the core, which may reflect oxygen availability. However, the total yeast and mold counts in the **rind** dropped dramatically in week 12 of ripening. Presumptive *Lactobacillus* (Rogosa agar) counts showed similar trends. The LAB (MRS agar), presumptive *Staphylococcus* (BP agar) and presumptive *Enterococcus* (KFSA) counts all showed a similar trend, being low in the raw milk, increasing after salting and with a peak in counts seen in the **rind** at 9 weeks, with core levels much lower; this difference did not appear to be related to a pH difference between **rind** and core samples at this stage and again may reflect differences in oxygen availability. These bacterial groups are all recognized as moderately halotolerant and hence are likely to increase after salt addition through reduction in competition with more halo-sensitive species. The mature cheese showed a lesser or no difference between **rind** and core for these counts despite a greater pH difference between the two samples (Table 1).

331 ***Characterization of Raw Milk and Matured Cheese Bacterial Isolates***

Eighty **bacterial** isolates were taken from the matured cheese and raw milk culture plates and sequenced (Table 2). The 16S rRNA gene sequencing showed the presence of *Lactobacillus curvatus*, *Staphylococcus gallinarum*, *Staphylococcus devriesei*, *Microbacterium* sp., *Sphingobacterium* sp., *Mycetocola* sp. and *Brevundimonas* sp. only in the raw milk sample. Conversely, *Enterococcus faecalis* and *Proteus* sp. were found only in the matured cheese. *Kocuria* sp. was detected in the milk and the core of the cheese samples, but not in the **rind**. *Lactococcus lactis* subsp. *lactis*

339 and *Lactobacillus plantarum* were found in all samples. All isolates, except *Proteus* sp.,
340 could grow at 10°C indicating they could grow during the early ripening step of this
341 cheese which is carried out at around 10°C. The Gram positive coccal species (with the
342 exception of *S. gallinarum*) showed growth at 40°C together with *Lactobacillus curvatus*
343 showing that the isolates could grow during the initial milk heat treatment prior to starter
344 culture addition and fermentation step which is carried out at 37-40°C.

345 It is notable that the M17 medium showed poor selectivity with a range of other
346 Gram positive and Gram negative genera isolated from this medium (Table 2). This
347 medium is widely used for isolating and enumerating *Lactococcus*, however, the counts
348 on M17 cannot be considered to reflect the levels of *Lactococcus* accurately. In contrast,
349 Rogosa agar, which is used for the enumeration of *Lactobacillus*, showed much greater
350 selectivity with *Lactobacillus plantarum* the main isolate, although *Kocuria* sp. were
351 also isolated on this medium. Notably *L. curvatus* was not isolated from Rogosa agar but
352 only on MRS agar; hence Rogosa agar may underestimate the *Lactobacillus* population
353 present.

355 **Bacterial Dynamics during Cheese Production**

356 We used three sets of primers (V3, V4V5 and V6V8) to amplify the variable
357 regions of 16S rRNA genes of the bacterial community from samples taken through
358 production of the cheese (Figure 1); bands identified by sequencing are shown marked.
359 The bacterial species detected varied at each production step and with each primer used,
360 with an increase in the diversity in the final cheese. The bacterial species in the rind
361 were more complex than those in the core (Figure 1, V4-V5, Lanes 10 and 11). In
362 general, *Lactococcus lactis* subsp. *lactis* and *Lactobacillus* sp. were the main

representatives in all samples with *Lactococcus* (band f) present initially in the raw milk through to the final cheese. *Lactobacillus plantarum* appeared at the post milling and salting stages (Figure 1, V3, Lane 5), as suggested by culturing, and was still detected in the fully ripened cheese (Figure 1, V3, Lane 11). *Staphylococcus equorum* and *Enterococcus durans* were found in the **rind** of a pre-piercing sample but not in the core and veins (Figure 1, V3, Lanes 6 and 7). Then, after cheese piercing, these species were found in all parts of the cheese, suggesting redistribution by the piercing process (Figure 1, V3, Lanes 8 and 9). However, these species only survived in the **rind** in the final product. Similarly, bands corresponding to *Acinetobacter* sp., *Alkalibacterium* sp. and *Corynebacterium casei* were found in the **rind** pre-piercing (Figure 1, V6V8, Lane 7), but, of these, only *Acinetobacter* sp. was detected in the core (Figure 1, V6V8, Lane 6). All three were present in the core post-piercing with the first two species still detected in the **rind** post-piercing and in the **rind** of the mature cheese (Figure 1, V6V8, Lanes 9 and 11). However, only *Acinetobacter* was detected in the core of the mature cheese suggesting an influence of pH or oxygen levels. *Lactobacillus casei/paracasei*, *Enterococcus faecalis* and *Brevibacterium* sp. were only found in the **rind** of the matured cheese suggesting surface introduction or development of these populations occurs late in production.

A comparison between the MRS, Rogosa and KFSA cultured populations with the uncultured populations from the mature cheese **is** shown in **Figure 2**. Mostly we found the same genera by direct DNA analysis of the culture plates as we identified as purified isolates (Table 2), although the exact species identified showed some variation. In particular *Enterococcus durans* and *Staphylococcus equorum* were found in samples grown on KFSA agar (Figure 2, Lane 8) and *Lactobacillus casei/paracasei* from MRS

387 and Rogosa agars (Figure 2, Lanes 6 and 7), although these species were not evident
388 from analysis of the DNA extracted directly from the respective cheese samples,
389 suggesting these species were in very low numbers in the cheese itself. *Lactococcus*
390 *lactis* was detected in the DNA sample produced by direct extraction from the cheese
391 and was also isolated from M17 and other agars (Table 2). This demonstrated that live
392 *Lactococcus lactis* cells were still evident in the final cheese. Direct DNA analysis also
393 showed the presence of *Brevibacterium* in the mature cheese rind which was not shown
394 by any culture plate analysis.

395

396 ***PFGE Profiles of Lactococcus lactis Isolates***

397 Twenty-six *Lactococcus lactis* isolates which were obtained at different stages of
398 cheese production and confirmed by 16S sequencing (Table 2) and three *Lactococcus*
399 *lactis* controls were subjected to *Sma*I restriction and PFGE profiling to determine strain
400 relatedness. The *Sma*I PFGE profiles are shown in Figure 3 together with a dendrogram
401 showing relatedness of the patterns. *Lactococcus lactis* isolates produced clear profiles
402 and were differentiated into three non-overlapping clusters with two out rider strains at
403 40% similarity. All isolates from the powdered starter culture were clustered together
404 with eight isolates from the liquid starter culture, one isolate from the cheese rind
405 (CRB11) and *Lactococcus lactis* subsp. *cremoris* NCIMB 8662 (Cluster 1). Another
406 isolate from the cheese rind (CRB10) clustered with isolates from the liquid starter
407 culture (Cluster 2). Two isolates found in the liquid starter culture were similar to
408 *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* NCIMB 10484 (Cluster 3), but not
409 similar with any isolates from the cheese core and veins or rind. Two isolates found in
410 the core and veins of mature cheese (COB20 and COB6) had profiles different from

those obtained from the liquid and powdered starter culture isolates. One of these isolates had a similar profile with *Lactococcus lactis* subsp. *lactis* NCIMB 6681. This suggests the *Lactococcus lactis* population originated from a number of sources.

414

415 DISCUSSION

416 In the present study, we have examined the bacterial species present during the
417 production of a raw milk mold-ripened cheese by using traditional culturing methods
418 and PCR-DGGE analysis of the variable V3, V4V5 and V6V8 regions of the 16S rRNA
419 genes. This method has previously been used successfully to identify the microbial
420 communities in Artisanal Sicilian (Randazzo et al., 2002), Stilton (Ercolini et al., 2003),
421 Mozzarella (Ercolini et al., 2004), Cabrales (Flórez and Mayo, 2006), Domiati (El-
422 Baradei et al., 2007) and Lighvan cheeses (Kafili et al., 2009). The use of a non-culture
423 based approach was chosen to allow all bacterial components present through the cheese
424 production to be identified although these may be in low number or may be cells not
425 readily culturable. Initially three different primer sets were used (V3, V4-5 and V6-V8)
426 to examine the bacterial diversity present as there have been reports of differing
427 sensitivity of these for different bacterial groups (Lorbeg et al., 2009). However, in
428 further analysis, only the V3 primer was used. This primer was chosen because V3 was
429 the best target for discriminating *Lactococcus lactis* whereas, when using V4-V5, this
430 species was detected in a triplet banding pattern which could lead to a bias in
431 interpretation (Figure 1). It is well known that some bacteria produce more than one
432 band because a microorganism can contain multiple rDNA copies of the target gene that
433 are heterogeneous in sequence (Florez and Mayo, 2006; Kang et al., 2010) and
434 differences in primer target may or may not identify these regions.

435 Compared to Stilton cheese (Ercolini et al., 2003), the viable counts of bacterial
436 groups of this raw milk cheese were lower on M17, MRS, Rogosa and NA agars. A
437 diverse microbiota including *Lactobacillus*, *Enterococcus* and *Staphylococcus* species
438 was culturable from the mature cheese, with *Lactococcus lactis* more readily recoverable
439 by culture than was shown from Stilton cheese. This could reflect a differing
440 *Lactococcus* composition between the two cheeses, as the raw milk used contained an
441 existing population which may have differing survival properties to the added starter
442 strains used for both cheeses. The initial mixed *Lactococcus lactis* community seen in
443 the raw milk by DGGE analysis was quite different from the final community seen in the
444 fully ripened cheese, suggesting a selection through processing. There were at least three
445 bands we identified in the V4V5 DGGE profile as *Lactococcus lactis* in the starter
446 (consistent with the three known sub-species present) and only one band of *Lactococcus*
447 *lactis* we identified in the raw milk; the three *Lactococcus lactis* bands were found
448 through the further process. However, only one band of *Lactococcus lactis* which was
449 identified both in milk and starter culture was on the final cheese, again suggesting
450 differing strain characteristics. From the cultured bacteria (Table 2), we identified the
451 *Lactococcus* isolates from the raw milk and all parts of the matured cheese as
452 *Lactococcus lactis* subsp. *lactis*. The source of the *L. lactis* subsp. *lactis* in the final
453 mature cheese in both core+veins and rind was examined by comparison of the mature
454 cheese isolates with isolates obtained from the starter cultures using *Sma*I PFGE. Whilst
455 isolates from the rind showed similarity to starter cultures used in the production,
456 particularly *Lactococcus lactis* subsp. *cremoris*, the isolates in the core and veins were
457 quite different. Rind isolates could have been reintroduced to the surface from handling
458 during ripening (more likely as strains matching two different starter sources were

459 evident), whereas isolates in the core and viens are more likely to have been present in
460 the milk and survived the fermentation process. This is significant as the latter may
461 have novel characteristics important for product quality and could be potential starter
462 cultures for this cheese production.

463 *Lactobacillus* species were cultured from the milk and were detected through the
464 process into the mature cheese by V3 and V4V5 amplification. However, whilst
465 *Lactobacillus plantarum* was isolated by culture and identified by DGGE analysis
466 through the entire process, *Lactobacillus curvatus* was isolated only in the raw milk and
467 was not detected through the processing stages which was an interesting finding because
468 it's presence is related to undesirable biofilm formation (Somers et al., 2001) and gas
469 production (Porcellato et al., 2015). In contrast, *Lactobacillus casei/paracasei* was
470 detected by V3 DGGE only on culture plates from the mature cheese. Thus
471 *Lactobacillus plantarum* in the matured cheese is likely to have originated from the raw
472 milk, but the source of *Lactobacillus casei/paracasei* is unclear. *Lactobacillus*
473 *plantarum* has an important role not only in flavor development (Amarita et al., 2001),
474 but has also been shown to inhibit *Listeria monocytogenes* in a smear-surface soft cheese
475 (Ennahar et al., 1998), and *Staphylococcus aureus* as well as *Salmonella* Typhimurium
476 in Montasio cheese (Stecchini et al., 1991). Its presence in both the core and the rind of
477 the mature cheese could therefore constitute an important biocontrol aspect, particularly
478 in a raw milk cheese where pathogens are not eliminated by pasteurization.

479 *Enterococcus faecalis* and *Enterococcus durans* have previously been reported in
480 cheeses (Ercolini et al., 2003; Delcenserie et al., 2014; Bulajic et al., 2015), however,
481 their presence in cheese is controversial. According to Gelsomino et al. (2001),
482 *Enterococcus faecalis* in cheese is usually assumed to be from fecal contamination and

its presence is undesirable because it can cause urinary tract infections in immune compromised patients (Coque et al., 1996). A study on enterococci in milk products (Gimenez-Pereira, 2005) revealed that enterococci strains from food are generally free from toxigenic potential in humans, which is different from enterococci strains from clinical sources, although these authors advised that the enterococcal levels in milk products should not exceed 10^7 cfu/g and good manufacturing practices were needed, specifically in the ripening period when high enterococcal levels were detected. On the other hand, this species contributes to taste and flavor development through proteolysis, lipolysis and citrate breakdown (Moreno et al., 2006). It is noticeable that we detected *Enterococcus faecalis* in the mature raw milk blue-veined cheese both by DGGE and culturing. We found no evidence of these organisms at other processing stages which suggests these populations may develop towards the end of the ripening period when the pH rises.

Staphylococcus equorum in contrast was found in the rind pre-piercing when the cheese was ripened at 21°C and introduced into the core by the piercing process, although it could only be cultured from culture plates of the rind of the mature cheese. This suggests this species is pH sensitive, as the core pre-piercing was pH 3.6 in comparison to the rind pH of 4.72 and the organism appears not to have survived exposure to this low pH, resulting in its subsequent absence from the core and vein samples of the mature cheese, even though their final pH was higher (pH 6.36). These observations support the finding that the growth of *Staphylococcus equorum* could be increased by increasing the pH from 4.0 to 6.0 and increasing the temperature from 10 to 26°C (Søndergaard and Stahnke, 2002). *Staphylococcus equorum* is a well-known starter culture in traditional sausage and ham production (Leroy et al., 2009; Landeta et

al., 2011). It could inhibit *Listeria monocytogenes* (Carnio et al., 2000) and, in Tilsit cheese, the combination of *Staphylococcus equorum* and *Debaryomyces hansenii* inhibited the growth of undesirable mold (Bockelmann, 2002). The other *Staphylococcus* found was *Staphylococcus gallinarum*. It was detected in the raw milk sample only (Table 2) indicating that this bacterium could not survive the fermentation process. *Staphylococcus gallinarum* was previously reported from chickens and a pheasant (Devriese et al., 1983) although it has been isolated from both healthy humans (Ohara-Nemoto et al., 2008) and clinical patients (Morfin-Otero et al., 2012).

In Stilton and other mold-ripened cheeses, the pH of the outer rind is usually higher than that of the inner regions indicating greater mold growth in the rind than in the core, as would be expected for these aerobic organisms. During fermentation, LAB produce acid causing the pH to drop, but as the molds grow, these break down proteins to produce ammonia and utilize the lactic acid, so the pH rises again. From our preliminary findings, the pH of the rind of three commercial Stilton cheeses was in the range 6.5 to 7.1 (unpublished data) while the pH of the raw milk cheese rind was at a higher level of pH 8.21. It was surprising therefore that the RBCA counts from the rind were lower than those of the core. This may represent a difference in the mold species growing or the extent to which they grow during the ripening period. The evaluation of yeast and fungal isolates was not part of this study but in Stilton these have been shown to be important components for final cheese characteristics (Gkatzionis et al., 2013, 2014).

We also noted that the bacterial diversity of the cheese was more complex than that reported previously for Stilton which is made with pasteurized milk (Ercolini et al., 2003); this process brings about changes in protein conformation and denaturation that

531 may have an impact on the species which may grow as well as removing the heat
532 sensitive components of the microbiota. Amongst the raw milk cheese bacterial
533 community we identified *Brevibacterium* sp., *Halomonas* sp., *Acinetobacter* sp.,
534 *Alkalibacterium* sp. and *Corynebacterium casei*. These microorganisms are salt-tolerant,
535 less acid-tolerant and have been found in the smear of surface-ripened cheeses where
536 they contribute to the sensory characteristics produced (Rattray and Fox, 1999; Maoz et
537 al., 2003; Mounier et al., 2005, 2007). The presence of *Halomonas* is likely to be from
538 salt addition and these appear to have grown optimally during ripening when
539 presumably a suitable environment had been reached. The presence of *Halomonas* sp. is
540 considered an indicator of process hygiene (Maoz et al., 2003), however it is also found
541 in smear ripened cheese (Mounier et al., 2005), and the influence of *Halomonas* sp. on
542 final cheese characteristics would warrant further study. *Alkalibacterium* has previously
543 been reported from mold-ripened cheeses with sea salt a suggested route of introduction
544 (Ishikawa et al., 2006). The development of an alkaline pH in the mature cheese rind
545 would explain the late isolation of this moderately halophilic alkaliphile from the mature
546 cheese and its absence from Stilton, where the rind pH is neutral. Species of this genus
547 produce organic acids (lactic, formic and acetic) and ethanol from glucose fermentation
548 (Ishikawa et al., 2009) and so could be active contributors to matured cheese
549 characteristics. However cultural isolation of this organism would require more
550 specialist conditions than used here (Ishikawa et al., 2006), indicating the value of the
551 non-culture based approach. *Brevibacterium* and *Corynebacterium*, although widely
552 found in cheese production, are genera associated with the skin and may have been
553 introduced to the cheese surface during handling of the cheese for turning. This concurs
554 with these being found only associated with the rind. *Acinetobacter* species have been

555 isolated from raw cows' milk and raw cows' milk cheeses (Rafei et al., 2015),
556 suggesting this species was derived from the raw milk used in production.

557 All these surface-ripening bacteria were detected using different amplimers,
558 demonstrating the value of analyzing 16S regions of mixed bacterial populations with
559 more than one primer set. This could introduce a bias in the interpretation of 16S rDNA
560 sequencing results depending on the primer set used. Most metagenomics studies of
561 cheese populations using new generation sequencing focus on targeting 16S rRNA genes
562 using universal primers and so primer bias cannot be discounted, although the high
563 number of reads may be favorable for identifying species present at low levels (Bokulich
564 and Mills, 2012).

565

566 CONCLUSIONS

567 The bacterial composition of this raw milk blue-veined cheese was more
568 complex than that of Stilton cheese. These bacteria are likely to have originated from the
569 raw milk, the salt and handling. We also showed how the piercing process can introduce
570 surface bacteria into the core of the cheese during the ripening process, although
571 survival in the core may be dependent on the pH conditions at the time of introduction.
572 *Lactobacillus casei/paracasei*, *Staphylococcus equorum*, *Bacillus* sp., *Brevibacterium*
573 sp., *Halomonas* sp., *Acinetobacter* sp., *Alkalibacterium* sp. and *Corynebacterium casei*
574 were only found by molecular methods. However, using different primers to analyze the
575 16S regions picked up different groups showing the advantage of using various primers
576 to examine mixed bacterial population. Also, culturing was still valuable as it
577 demonstrated viability of particular groups and detected a large number of raw milk

578 **microbiota.** It was notable that *Lactococcus lactis* **subsp.** *lactis* was viable in the core
 579 and **rind** of the final cheese **and some of these isolates originated from the raw milk.**

580

581

ACKNOWLEDGEMENTS

582 The first author gratefully thanks The University of Nottingham and DG-RSTHE
 583 (Directorate General of Resources for Science, Technology and Higher Education;
 584 formerly known as DGHE) Ministry of Research, Technology and Higher Education of
 585 Indonesia for financial support. The authors would also like to thank Randolph Hodgson
 586 and Joe Schneider from Stichelton Dairy Ltd. for their assistance in this study and
 587 allowing sampling of the cheese production unit.

588

589

REFERENCES

- 590 Amarita, F., T. Requena, G. Taborda, L. Amigo, and C. Pelaez. 2001. *Lactobacillus*
 591 *casei* and *Lactobacillus plantarum* initiate catabolism of methionine by
 592 transamination. *J. Appl. Microbiol.* 90:971–978. [http://dx.doi.org/10.1046/j.1365-](http://dx.doi.org/10.1046/j.1365-2672.2001.01331.x)
 593 [2672.2001.01331.x](http://dx.doi.org/10.1046/j.1365-2672.2001.01331.x).
- 594 Bockelmann, W. 2002. Development of defined surface starter cultures for the ripening
 595 of smear cheeses. *Int. Dairy J.* 12:123–131. [http://dx.doi.org/10.1016/S0958-](http://dx.doi.org/10.1016/S0958-6946(01)00152-2)
 596 [6946\(01\)00152-2](http://dx.doi.org/10.1016/S0958-6946(01)00152-2).
- 597 **Bokulich, N. A., and D. A. Mills. 2012. Next-generation approaches to the microbial**
 598 **ecology of food fermentations. *BMB Rep.* 45:377-89.**
 599 **<http://dx.doi.org/10.5483/BMBRep.2012.45.7.148>.**
- 600 Bulajic, S., Z. Tambur, D. Opacic, B. Miljkovic-Selimovic, R. Doder, and D. Cenic-

- 601 Milosevic. 2015. Characterization of antibiotic resistance phenotypes and resistance
602 genes in *Enterococcus spp.* isolated from cheeses. *Arch. Biol. Sci.* 67:139–146.
603 <http://dx.doi.org/10.2298/ABS140426016B>.
- 604 Carnio, M.C., A. Hölzel, M. Rudolf, T. Henle, G. Jung, and S. Scherer. 2000. The
605 macrocyclic peptide antibiotic micrococcin P1 is secreted by the food-borne
606 bacterium *Staphylococcus equorum* WS 2733 and inhibits *Listeria monocytogenes*
607 on soft cheese. *Appl. Environ. Microbiol.* 66:2378–2384.
608 <http://dx.doi.org/10.1128/AEM.66.6.2378-2384.2000>.
- 609 Cocolin, L., M. Manzano, C. Cantoni, and G. Comi. 2001. Denaturing Gradient gel
610 electrophoresis analysis of the 16S rRNA gene V1 Region to monitor dynamic
611 changes in the bacterial population during fermentation of italian sausages. *Appl.*
612 *Environ. Microbiol.* 67:5113–5121. [http://dx.doi.org/10.1128/AEM.67.11.5113-](http://dx.doi.org/10.1128/AEM.67.11.5113-5121.2001)
613 [5121.2001](http://dx.doi.org/10.1128/AEM.67.11.5113-5121.2001).
- 614 Conte, A., I. Brescia, and M.A. Del Nobile. 2011. Lysozyme/EDTA disodium salt and
615 modified-atmosphere packaging to prolong the shelf life of Burrata cheese. *J. Dairy*
616 *Sci.* 94:5289–5297. <http://dx.doi.org/10.3168/jds.2010-3961>.
- 617 Coque, T., J. Tomayko, S. Ricke, P. Okhyusen, and B. Murray. 1996. Vancomycin-
618 resistant enterococci from nosocomial, community, and animal sources in the
619 United States. *Antimicrob. Agents Chemother.* 40:2605–2609.
- 620 Delcenserie, V., B. Taminiau, L. Delhalle, C. Nezer, P. Doyen, S. Crevecœur, D.
621 Roussey, N. Korsak, and G. Daube. 2014. Microbiota characterization of a Belgian
622 protected designation of origin cheese, Herve cheese, using metagenomic analysis.
623 *J. Dairy Sci.* 97:6046–6056. <http://dx.doi.org/10.3168/jds.2014-8225>.
- 624 Devriese, L.A., B. Poutrel, R. Kilpper-Balz, and K.H. Schleifer. 1983. *Staphylococcus*

- 625 *gallinarum* and *Staphylococcus caprae*, two new species from animals. *Int. J. Syst.*
626 *Bacteriol.* 33:480–486. <http://dx.doi.org/10.1099/00207713-33-3-480>.
- 627 El-Baradei, G., A. Delacroix-Buchet, and J. -C. Ogier. 2007. Biodiversity of bacterial
628 ecosystems in traditional Egyptian Domiati cheese. *Appl. Environ. Microbiol.*
629 73:1248–1255. <http://dx.doi.org/10.1128/AEM.01667-06>.
- 630 Ercolini, D., P. J. Hill, and C. E. R. Dodd. 2003. Bacterial community structure and
631 location in Stilton cheese. *Appl. Environ. Microbiol.* 69:3540–3548.
632 <http://dx.doi.org/10.1128/AEM.69.6.3540-3548.2003>.
- 633 Ercolini, D., G. Mauriello, G. Blaiotta, G. Moschetti, and S. Coppola. 2004. PCR-DGGE
634 Fingerprints of microbial succession during a manufacture of traditional water
635 buffalo Mozzarella cheese. *J. Appl. Microbiol.* 96:263–270.
636 <http://dx.doi.org/10.1046/j.1365-2672.2003.02146.x>
- 637 Flórez, A. B., and B. Mayo. 2006. PCR–DGGE as a tool for characterizing dominant
638 microbial populations in the spanish blue-veined Cabrales cheese. *Int. Dairy J.*
639 16:1205–1210. <http://dx.doi.org/10.1016/j.idairyj.2005.11.008>.
- 640 McSweeney, P. L. H. and P. F. Fox. 2004. Metabolism of residual lactose and of
641 lactate and citrate. Pages 361–371 in *Cheese: Chemistry, Physics and Microbiology*
642 Vol. 1. P. F. Fox, P. L. H. McSweeney, T. M. Cogan, T. P. Guinee, ed. Academic
643 Press, London, UK.
- 644 Gelsomino, R., M. Vancanneyt, S. Condon, J. Swings, and T. M. Cogan. 2001.
645 Enterococcal diversity in the environment of an irish cheddar-type cheesemaking
646 factory. *Int. J. Food Microbiol.* 71:177–188. [http://dx.doi.org/10.1016/S0168-](http://dx.doi.org/10.1016/S0168-1605(01)00620-1)
647 1605(01)00620-1.
- 648 Gimenez-Pereira, M.L. 2005. Enterococci in milk products. [Master Thesis]. Massey

- University, New Zealand. <http://dx.doi.org/10.3109/10408419109113508>.
- Gkatzionis, K., L. Hewson, T. Hollowood, J. Hort, C.E.R. Dodd, and R.S.T. Linfoth. 2013. Effect of *Yarrowia lipolytica* on blue cheese odour development: flash profile sensory evaluation of microbiological models and cheeses. *Int. Dairy J.* 30:8–13. <http://dx.doi.org/10.1016/j.idairyj.2012.11.010>.
- Gkatzionis, K., D. Yunita, R.S.T. Linfoth, M. Dickinson, and C.E.R. Dodd. 2014. Diversity and activities of yeasts from different parts of a Stilton cheese. *Int. J. Food Microbiol.* 177:109–116. <http://dx.doi.org/10.1016/j.ijfoodmicro.2014.02.016>.
- Gori, K., H.D. Mortensen, N. Arneborg, and L. Jespersen. 2007. Ammonia production and its possible role as a mediator of communication for *Debaryomyces hansenii* and other cheese-relevant yeast Species. *J. Dairy Sci.* 90:5032–5041. doi:10.3168/jds.2006-750.
- Ishikawa, M., K. Kodama, H. Yasuda, A. Okamoto-Kainuma, K. Koizumi, and K. Yamasato. 2006. Presence of halophilic and alkaliphilic lactic acid bacteria in various cheeses. *Lett. Appl. Microbiol.* 44:308–313. <http://dx.doi.org/10.1111/j.1472-765X.2006.02073.x>.
- Ishikawa, M., S. Tanasupawat, K. Nakajima, H. Kanamori, S. Ishizaki, K. Kodama, A. Okamoto-Kainuma, Y. Koizumi, Y. Yamamoto, and K. Yamasato. 2009. *Alkalibacterium thalassium* sp. nov., *Alkalibacterium pelagium* sp. nov., *Alkalibacterium putridalgicola* sp. nov. and *Alkalibacterium kapii* sp. nov., slightly halophilic and alkaliphilic marine lactic acid bacteria isolated from marine organisms and salted foods collected in Japan and Thailand. *Int. J. Syst. Evol. Microbiol.* 59:1215–1226. <http://dx.doi.org/10.1099/ijss.0.65602-0>.
- Kafili, T., S. H. Razavi, Z. E. Djomeh, M. R. Naghavi, P. Álvarez-Martín, and B. Mayo.

2009. Microbial characterization of Iranian traditional Lighvan cheese over manufacturing and ripening via culturing and PCR-DGGE analysis: identification and typing of dominant lactobacilli. *Eur. Food Res. Technol.* 229:83–92. <http://dx.doi.org/10.1007/s00217-009-1028-x>.
- Kang, Y.J., J. Cheng, L.J. Mei, J. Hu, Z. Piao, and S.X. Yin. 2010. Multiple copies of 16S rRNA gene affect the restriction patterns and DGGE profile revealed by analysis of genome database. *Microbiology.* 79:655–662. <http://dx.doi.org/10.1134/S0026261710050103>.
- Lafarge, V. L., C. Lahellec. (2000). Hygiene Aspects: Pathogenic Bacteria. In: *Cheese Making from Science to Quality Assurance* 2nd Edition, A. Eck and J. C. Gillis (Eds.). Intercept Ltd., United Kingdom.
- Landeta, G., I. Reverón, A. V Carrascosa, B. de las Rivas, and R. Muñoz. 2011. Use of recA gene sequence analysis for the identification of *Staphylococcus equorum* strains predominant on dry-cured hams. *Food Microbiol.* 28:1205–1210. <http://dx.doi.org/10.1016/j.fm.2011.04.006>.
- Leroy, S., I. Lebert, J. -P. Chacornac, P. Chavant, T. Bernardi, and R. Talon. 2009. Genetic diversity and biofilm formation of *Staphylococcus equorum* isolated from naturally fermented sausages and their manufacturing environment. *Int. J. Food Microbiol.* 134:46–51. <http://dx.doi.org/10.1016/j.ijfoodmicro.2008.12.012>.
- Lorbeg, P.M., A.Č. Majhenič, and I. Rogelj. 2009. Evaluation of different primers for PCR-DGGE analysis of cheese-associated enterococci. *J. Dairy Res.* 76:265–271. <http://dx.doi.org/10.1017/S0022029909003902>.
- Maoz, A., R. Mayr, and S. Scherer. 2003. Temporal stability and biodiversity of two complex antilisterial cheese-ripening microbial consortia. *Appl. Environ. Microbiol.*

- 697 69:4012–4018. <http://dx.doi.org/10.1128/AEM.69.7.4012-4018.2003>.
- 698 Mayo, B., C.T.C.C. Rachid, A. Alegría, A.M.O. Leite, R.S. Peixoto, and S. Delgado.
- 699 2014. Impact of next generation sequencing techniques in food microbiology. *Curr.*
- 700 *Genomics*. 15:1–17. <http://dx.doi.org/10.2174/1389202915666140616233211>.
- 701 Morfin-Otero, R., M. A. Martínez-Vázquez, D. López, E. Rodríguez-Noriega, and E.
- 702 Garza-González. 2012. Isolation of rare coagulase-negative isolates in
- 703 immunocompromised patients: *Staphylococcus gallinarum*, *Staphylococcus*
- 704 *pettenkoferi* and *Staphylococcus pasteurii*. *Ann. Clin. Lab. Sci.* 42: 182-185.
- 705 Montel, M.-C., S. Buchin, A. Mallet, C. Delbes-Paus, D. a Vuitton, N. Desmaures, and
- 706 F. Berthier. 2014. Traditional cheeses: rich and diverse microbiota with associated
- 707 benefits. *Int. J. Food Microbiol.* 177:136–154.
- 708 <http://dx.doi.org/10.1016/j.ijfoodmicro.2014.02.019>.
- 709 Moreno, M. R. F., P. Sarantinopoulos, E. Tsakalidou, and L. De Vuyst. 2006. The role
- 710 and application of enterococci in food and health. *Int. J. Food Microbiol.* 106:1–24.
- 711 <http://dx.doi.org/10.1016/j.ijfoodmicro.2005.06.026>.
- 712 Mounier, J., R. Gelsomino, S. Goerges, M. Vancanneyt, K. Vandemeulebroecke, B.
- 713 Hoste, S. Scherer, J. Swings, G.F. Fitzgerald, and T.M. Cogan. 2005. Surface
- 714 microflora of four smear-ripened cheeses. *Appl. Environ. Microbiol.* 71:6489–6500.
- 715 <http://dx.doi.org/10.1128/AEM.71.11.6489-6500.2005>.
- 716 Mounier, J., F. Irlinger, M.-N. Leclercq-Perlat, A.-S. Sarthou, H.-E. Spinnler, G.F.
- 717 Fitzgerald, and T.M. Cogan. 2006. Growth and colour development of some
- 718 surface ripening bacteria with *Debaryomyces hansenii* on aseptic cheese curd. *J.*
- 719 *Dairy Res.* 73:441–448. doi:10.1017/S0022029906001919.
- 720 Mounier, J., M. C. Rea, P. M. O'Connor, G. F. Fitzgerald, and T. M. Cogan. 2007.

- 721 Growth characteristics of *Brevibacterium*, *Corynebacterium*, *Microbacterium*, and
722 *Staphylococcus spp.* isolated from surface-ripened cheese. *Appl. Environ.*
723 *Microbiol.* 73:7732–7739. <http://dx.doi.org/10.1128/AEM.01260-07>.
- 724 Muyzer, G., E. C. D. E. Waal, and A.G. Uitterlinden. 1993. Profiling of complex
725 microbial populations by denaturing gradient gel electrophoresis analysis of
726 polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ.*
727 *Microbiol.* 59:695–700.
- 728 Nübel, U., B. Engelen, A. Felske, J. Snaidr, A. Wieshuber, R. I. Amann, W. Ludwig,
729 and H. Backhaus. 1996. Sequence heterogeneities of genes encoding 16S rRNAs in
730 *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *J.*
731 *Bacteriol.* 178:5636–5643. <http://dx.doi.org/10.1128/jb.178.19.5636-5643.1996>.
- 732 Obodai, M. A. B. 2006. Characterisation of the dominant microbiota in Nyamie, a
733 Ghanaian fermented milk product. PhD Thesis. The University of Nottingham,
734 United Kingdom.
- 735 Ohara-Nemoto, Y., H. Haraga, S. Kimura, and T. K. Nemoto. 2008. Occurrence of
736 staphylococci in the oral cavities of healthy adults and nasal oral trafficking of the
737 bacteria". *J. Med. Microbiol.* 57: 95–99. <http://dx.doi.org/10.1099/jmm.0.47561-0>.
- 738 Porcellato, D., M.E. Johnson, K. Houck, S.B. Skeie, D.A. Mills, K.M. Kalanetra, and
739 J.L. Steele. 2015. Potential of *Lactobacillus curvatus* LFC1 to produce slits in
740 cheddar cheese. *Food Microbiol.* 49:65–73.
741 <http://dx.doi.org/10.1016/j.fm.2015.01.015>.
- 742 Price, E. J., R. S. T. Linforth, C. E. R. Dodd, C. A. Phillips, L. Hewson, J. Hort, and K.
743 Gkatzionis. 2014. Study of the influence of yeast inoculum concentration (*Yarrowia*
744 *lipolytica* and *Kluyveromyces lactis*) on blue cheese aroma development using

- 745 microbiological models. *Food Chem.* 145:464–472.
746 <http://dx.doi.org/10.1016/j.foodchem.2013.08.081>.
- 747 Rafei, R., M. Hamze, H. Pailhories, M. Eveillard, L. Marsollier, M. L. Joly-Guillou, F.
748 Dabboussi, and M. Kempf. 2015. Extrahuman epidemiology of *Acinetobacter*
749 *baumannii* in Lebanon. *Appl. Environ. Microbiol.* 81:2359–2367.
750 <http://dx.doi.org/10.1128/AEM.03824-14>.
- 751 Randazzo, C. L., S. Torriani, A. D. L. Akkermans, W. M. de Vos, and E. E. Vaughan.
752 2002. Diversity, dynamics, and activity of bacterial communities during production
753 of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. *Appl. Environ.*
754 *Microbiol.* 68:1882–1892. <http://dx.doi.org/10.1128/AEM.68.4.1882-1892.2002>.
- 755 Rattray, F. P., and P. F. Fox. 1999. Aspects of enzymology and biochemical properties
756 of *Brevibacterium linens* relevant to cheese ripening: a review. *J. Dairy Sci.*
757 82:891–909. [http://dx.doi.org/10.3168/jds.S0022-0302\(99\)75308-7](http://dx.doi.org/10.3168/jds.S0022-0302(99)75308-7).
- 758 Schwieger, F., and C. C. Tebbe. 1998. A new approach to utilize PCR – polymorphism
759 for 16S rRNA gene-based microbial community analysis. *Appl. Environ. Microbiol.*
760 64:4870–4876.
- 761 Somers, E.B., M.E. Johnson, and A.C.L. Wong. 2001. Biofilm formation and
762 contamination of cheese by nonstarter lactic acid bacteria in the dairy environment.
763 *J. Dairy Sci.* 84:1926–1936. [http://dx.doi.org/10.3168/jds.S0022-0302\(01\)74634-6](http://dx.doi.org/10.3168/jds.S0022-0302(01)74634-6).
- 764 Søndergaard, A. K., and L. H. Stahnke. 2002. Growth and aroma production by
765 *Staphylococcus xylosus*, *S. carnosus* and *S. equorum*—a comparative study in model
766 systems. *Int. J. Food Microbiol.* 75:99–109. [http://dx.doi.org/10.1016/S0168-](http://dx.doi.org/10.1016/S0168-1605(01)00729-2)
767 [1605\(01\)00729-2](http://dx.doi.org/10.1016/S0168-1605(01)00729-2).

768 Stecchini, M. L., I. Sarais, and M. De Bertoldi. 1991. The influence of *Lactobacillus*
769 *plantarum* culture inoculation on the fate of *Staphylococcus aureus* and *Salmonella*
770 *typhimurium* in Montasio cheese. *Int. J. Food Microbiol.* 14:99–110.
771 [http://dx.doi.org/10.1016/0168-1605\(91\)90096-8](http://dx.doi.org/10.1016/0168-1605(91)90096-8).

For Peer Review

772 Figures and Tables

773 **Figure 1.** Comparison of bacterial DGGE profiles of PCR amplicons of community 16S
 774 rRNA genes from samples taken at different stages of raw milk blue-veined cheese
 775 production using primer sets for three different rRNA regions: V3; V4-5; V6-8. Samples
 776 in Lane: 1 and 2, Milk; 3, Starter culture; 4, Pre-milling; 5, Post-milling and salting; 6,
 777 Pre-piercing core and veins; 7, Pre-piercing **rind**; 8, Post-piercing core and veins; 9,
 778 Post-piercing **rind**; 10, Matured cheese (core and veins); 11, Matured cheese (**rind**).
 779 Bands identified by excision and sequencing: a, *Lactobacillus casei/paracasei*; b,
 780 *Lactobacillus plantarum*; c, *Staphylococcus equorum*; d, *Enterococcus durans*; e,
 781 *Enterococcus faecalis*; f, *Lactococcus lactis*; g, *Brevibacterium* sp.; h, *Acinetobacter* sp.;
 782 i, *Alkalibacterium* sp.; j, *Corynebacterium casei*; k, *Bacillus* sp.; l, *Halomonas* sp. Gel
 783 running conditions are given in material and methods.

784

785 **Figure 2.** DGGE profiles of PCR amplicons of V3 regions of community 16S rRNA
 786 genes from samples of mature raw milk blue-veined cheese and cultured cells from
 787 culture plates. Samples in Lane: 1, Mature cheese (core and veins; direct DNA
 788 extraction); 2, Cultured cells on MRS agar from mature cheese (core and veins); 3,
 789 Cultured cells on Rogosa agar from mature cheese (core and veins); 4, Cultured cells on
 790 KFSA agar from mature cheese (core and veins); 5, Mature cheese (**rind**; direct DNA
 791 extraction); 6, Cultured cells on MRS agar from mature cheese (**rind**); 7, Cultured cells
 792 on Rogosa agar from mature cheese (**rind**); 8, Cultured cells on KFSA agar from mature
 793 cheese (**rind**); 9, *Lactococcus lactis* NCIMB 9918; 10, *Lactococcus lactis* NCIMB 8763;
 794 11, *Lactobacillus plantarum* NCIMB 138914; 12, *Leuconostoc mesenteroides*; 13,

795 *Enterococcus faecalis* NCTC 775. Bands were identified by excision and sequencing.

796 Gel running conditions are given in material and methods.

797

798 **Figure 3.** Dendrogram and *Sma*I restriction patterns of *Lactococcus lactis*. NCIMB 6681

799 is the subspecies *lactis*, NCIMB 8662 is the subspecies *cremoris*, and NCIMB 10484 is

800 the subspecies *lactis* biovar *diacetylactis*. Clustering was performed by the UPGMA

801 method. The coefficient of similarity (40%) is indicated on the figure by the bold

802 vertical line.

For Peer Review

803 Table 1. Microbial counts^a at different stages of raw milk blue-veined cheese production.

Samples		pH	MRS (Lactic Acid Bacteria)	Rogosa (<i>Lactobacillus</i>)	M17 (<i>Lactococcus</i>)	BP (<i>Staphylococcus</i>)	KFSA (<i>Enterococcus</i>)	BHI (Total Aerobic Counts)	RBCA (Yeasts and Moulds)
Starter Cultures		4.47 (0.08) ^b	0.00 (0.00)	0.00 (0.00)	8.46 (0.01)	0.00 (0.00)	0.00 (0.00)	9.10 (0.05)	0.00 (0.00)
Raw Milk		6.65 (0.11)	2.76 (0.28)	0.00 (0.00)	4.00 (0.07)	2.51 (0.30)	2.18 (0.54)	4.06 (0.00)	0.00 (0.00)
Pre-Milling		4.80 (0.41)	5.26 (0.99)	0.00 (0.00)	9.00 (0.07)	4.51 (0.30)	3.84 (1.01)	9.06 (0.00)	0.00 (0.00)
Post Milling and Salting		4.80 (0.13)	7.61 (0.04)	7.58 (0.06)	8.73 (0.19)	5.88 (0.02)	7.51 (0.03)	8.72 (0.08)	4.88 (0.05)
Pre-Piercing (6 weeks)	Core	3.60 (0.77)	8.13 (0.02)	7.79 (0.23)	8.16 (0.05)	2.96 (0.46)	7.98 (0.04)	8.14 (0.01)	5.49 (0.07)
	Rind	4.72 (0.27)	8.02 (0.04)	7.97 (0.02)	11.32 (0.05)	4.83 (0.04)	7.99 (0.02)	11.43 (0.03)	5.90 (0.03)
Post Piercing (9 weeks)	Core + Veins	6.70 (0.58)	7.89 (0.06)	7.32 (0.04)	8.04 (0.04)	4.45 (0.26)	7.22 (0.05)	8.03 (0.16)	7.28 (0.01)
	Rind	6.32 (0.12)	10.70 (0.09)	7.08 (0.11)	11.00 (0.05)	10.60 (0.08)	10.68 (0.07)	11.28 (0.02)	9.99 (0.04)
Mature cheese (12 weeks)	Core + Veins	6.36 (0.24)	7.41 (0.01)	6.18 (0.04)	7.83 (0.06)	5.35 (0.06)	7.44 (0.03)	8.17 (0.21)	6.66 (0.07)
	Rind	8.21 (0.31)	6.90 (0.06)	4.85 (0.05)	8.65 (0.05)	7.03 (0.03)	7.09 (0.03)	8.92 (0.02)	4.53 (0.09)

804 ^alog₁₀ cfu/ml for milk and starter cultures; log₁₀ cfu/g for cheese samples.

805 ^bMean (Standard Deviation, SD).

806 Table 2. Bacteria detected in raw milk and matured raw milk blue-veined cheese using culturing methods.

Isolate identification by 16S rRNA sequencing	No. of Isolates (80)	Samples			Media (30°C)	Ability to grow at		Gram Staining	Cell Shape
		Raw Milk	Mature cheese Core and Veins	Mature cheese Rind		10°C	40°C		
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	33	+	+	+	M17, MRS, BHI	+	+	+	Cocci, small
<i>Lactobacillus plantarum</i>	4	+	+	+	MRS, KFSA, Rogosa	+	-	+	Rod, small
<i>Lactobacillus curvatus</i>	3	+	-	-	MRS	+	+	+	Rod, big
<i>Staphylococcus gallinarum</i>	3	+	-	-	MRS, M17	+	-	+	Cocci, big
<i>Staphylococcus devriesei</i>	2	+	-	-	M17	+	+	+	Cocci, big
<i>Microbacterium</i> sp.	1	+	-	-	M17	+	-	+	Rod, small
<i>Sphingobacterium</i> sp.	3	+	-	-	M17	+	-	-	Rod, very small
<i>Mycetocola</i> sp.	1	+	-	-	M17	+	-	+	Cocci, very small
<i>Brevundimonas</i> sp.	3	+	-	-	M17	+	-	-	Rod, very small
<i>Kocuria</i> sp.	13	+	+	-	M17, MRS, Rogosa	+	+	+	Cocci, big
<i>Enterococcus faecalis</i>	13	-	+	+	KFSA, M17	+	+	+	Cocci, small
<i>Proteus</i> sp.	1	-	-	+	BP, M17	-	-	-	Rod, very small

807 Except for Gram staining results + : Detected , - : Not detected.





